



TITLE:

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CITATION:

Takachi, Takayuki ...[et al]. Human T-cell leukemia virus type 1 Tax oncoprotein represses the expression of the BCL11B tumor suppressor in T-cells. Cancer Science 2015, 106(4): 461-465

ISSUE DATE:

2015-04

URL:

<http://hdl.handle.net/2433/215158>

RIGHT:

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Human T-cell leukemia virus type 1 Tax oncoprotein represses the expression of the BCL11B tumor suppressor in T-cells

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Key words

Adult T cell leukemia, BCL11B, HBZ, HTLV-1, Tax

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Funding Information

Ministry of Education, Culture, Sports, Science and Technology of Japan, Promotion of Niigata University Research Project, National Institutes of Health (CA100730).

Received September 28, 2014; Revised January 7, 2015;
Accepted January 17, 2015

Cancer Sci 106 (2015) 461–465

doi: 10.1111/cas.12618

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T cell leukemia (ATL), which is an aggressive form of T-cell malignancy. HTLV-1 oncoproteins, Tax and HBZ, play crucial roles in the immortalization of T-cells and/or leukemogenesis by dysregulating the cellular functions in the host. Recent studies show that HTLV-1-infected T-cells have reduced expression of the BCL11B tumor suppressor protein. In the present study, we explored whether Tax and/or HBZ play a role in downregulating BCL11B in HTLV-1-infected T-cells. Lentiviral transduction of Tax in a human T-cell line repressed the expression of BCL11B at both the protein and mRNA levels, whereas the transduction of HBZ had little effect on the expression. Tax mutants with a decreased activity for the NF- κ B, CREB or PDZ protein pathways still showed a reduced expression of the BCL11B protein, thereby implicating a different function of Tax in BCL11B downregulation. In addition, the HTLV-2 Tax2 protein reduced the BCL11B protein expression in T-cells. Seven HTLV-1-infected T-cell lines, including three ATL-derived cell lines, showed reduced BCL11B mRNA and protein expression relative to an uninfected T-cell line, and the greatest reductions were in the cells expressing Tax. Collectively, these results indicate that Tax is responsible for suppressing BCL11B protein expression in HTLV-1-infected T-cells; Tax-mediated repression of BCL11B is another mechanism that Tax uses to promote oncogenesis of HTLV-1-infected T-cells.

Human T-cell leukemia virus type 1 (HTLV-1) is a causative agent of adult T-cell leukemia (ATL), which is characterized as an aggressive mature T-cell leukemia. HTLV-1 has two oncoproteins, Tax and HTLV-1 bZIP factor (HBZ), both of which play crucial roles in persistent HTLV-1 infection and leukemogenesis.^(1–3) Tax by itself immortalizes primary human T-cells *in vitro*, and *tax*-transgenic mice develop various malignancies, including mature T-cell leukemia. To achieve these effects, Tax interacts with several cellular proteins involved in cell cycle regulation,^(4,5) apoptosis,⁽⁶⁾ genomic instability^(7,8) and DNA repair,⁽⁶⁾ and modulates their functions. For instance, Tax interacts with various transcription regulatory factors, including cAMP-responsive element-binding protein/activating transcription factor (CREB/ATF) and IKK, through which Tax activates the expression of a number of cellular genes.

Unlike Tax, HBZ is dispensable for T-cell immortalization by HTLV-1, but it was required for persistent HTLV-1 infection in an *in vivo* animal model.⁽⁹⁾ In addition, HBZ-transgenic mice develop mature T-cell lymphoma.⁽¹⁰⁾ HBZ also has multiple activities. For example, HBZ prevents apoptosis by promoting the anti-apoptotic function of FoxO3a.⁽¹¹⁾

HBZ upregulates the expression of the hTERT telomerase subunit gene,⁽¹²⁾ and inhibits the transcriptional activation of cellular genes mediated by c-Jun, CREB/ATF and RelA.^(13–15)

BCL11B is a transcriptional regulatory protein containing C2H2-type zinc fingers, and it is required for normal T-cell development.⁽¹⁶⁾ Moreover, BCL11B has been shown to act as a tumor suppressor gene in T-cell acute lymphoblastic leukemia (T-ALL). Genetic alterations of BCL11B, such as by chromosomal rearrangements, have been identified in several T-ALL patients.⁽¹⁷⁾ Intriguingly, HTLV-1-infected T-cells, including ATL cells, have been reported to have reduced BCL11B protein expression,⁽¹⁸⁾ but the mechanism remains to be elucidated. In the present study, we show that the HTLV-1 oncoprotein Tax is sufficient to downregulate BCL11B expression in T-cells.

Materials and Methods

Cell lines and culture condition. SLB-1, HUT-102, MT-2 and MT-4 are HTLV-1-transformed human T-cell lines. TL-OmI, KK-1 and KOB are HTLV-1-positive ATL patient-derived cell lines. Jurkat and MOLT-4 are HTLV-1-negative T-cell lines.

These human T-cell lines were cultured in RPMI1640 medium supplemented with 10% FBS, 4 mM L-glutamine and antibiotics (RPMI/10%FBS). Recombinant human IL-2 (Takeda Chemical Industries, Osaka, Japan) was added at 0.5 nM to the cultures of KK-1 and KOB cells. 293T cells, which are highly transfectable kidney-derived cells, were cultured in DMEM supplemented with 10% FBS, 4 mM L-glutamine and antibiotics.

Plasmids. CSII-EF-Tax-IRES and CSII-EF-Tax-IRES-sHBZ were the IRES-mediated bicistronic lentiviral vectors for Tax and Tax together with spliced-HBZ (sHBZ), respectively. CSII-EF-IRES-GFP was used as a control vector. The lentiviral expression vectors for HTLV-1 Tax, its mutants (Tax Δ C, TaxM22, Tax703, Tax[225–232]) and HTLV-2 Tax2B have been described previously.^(19,20) Tax(TTG) has a mutation from A to T at the initiation codon of *tax* gene, and, thus, it expresses the Tax transcript, but not its protein. Tax(TTG) cDNA was constructed by introducing mutation with the PCR. Next, the Tax(TTG) cDNA was cloned into the pENTR/D-TOPO plasmid (Invitrogen, Carlsbad, CA, USA), and then the cDNA was transferred into lentiviral vector CSII-EF-IRES-GFP-RfA⁽²⁰⁾ through an LR recombination reaction using LR clonase (Invitrogen). The expression vector pH β Pr-1-neo was used for the transient expression of Tax, Tax2B and its mutant proteins (Tax Δ C, TaxM22, Tax703) in Jurkat cells in order to perform a luciferase assay as described previously.^(21,22) Tax (225–232) protein is defective for the activation of the non-canonical NF- κ B/p100/p52 pathway, but it is active for the canonical NF- κ B pathway,⁽¹⁹⁾ and the expression vector was constructed by inserting the Tax(225–232) gene into the expression vector pH β Pr-1-neo. The transcriptional activity reporter plasmids, κ B-luc, CRE-luc and pGK/ β -gal, have all been described previously.^(22,23)

Lentivirus transduction. Recombinant lentiviruses were generated by transfecting pCAG-HIVgp, pCMV-VSV-G-RSV-Rev and the respective lentiviral vectors into 293T cells using FuGENE HD (Roche Diagnostic, Mannheim, Germany). Seventy-two hours after transfection, the culture supernatants were harvested, and were infected into Jurkat or MOLT-4 cells (4×10^5) at a final volume of 2 mL of RPMI/10%FBS containing 8 μ g/mL polybrene.

Quantitative real-time RT-PCR. Total RNA was isolated from cells using the NucleoSpin RNA II Kit (MACHEREY-NA-GEL; TaKaRa, Shiga, Japan), and RNA was reverse-transcribed using the PrimeScript RT reagent kit (TaKaRa). BCL11B cDNA fragments were amplified and analyzed by the real-time PCR assay performed using SYBR Green Real-Time PCR Mix (TOYOBO, Osaka, Japan) with a 25 μ L reaction volume. The quantity of BCL11B mRNA was normalized to the quantity of GAPDH mRNA.

Western blot analysis. The western blot analyses were carried out as described previously.⁽²⁴⁾ The primary antibodies used at the indicated dilutions were anti-BCL11B (1/1000),⁽¹⁶⁾ anti-BCL11B (1/1000) (Cell Signaling Technology, Beverly, MA, USA), anti-Tax (1/2000) (Tax7),⁽²⁵⁾ anti-Tax2,⁽²⁶⁾ anti- α -tubulin (DM1A) (1/1000) (Calbiochem, San Diego, CA, USA) and α -HBZ (1/1000).⁽⁹⁾ Horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG kappa light chain (BD Biosciences, San Jose, CA, USA) and HRP-conjugated goat anti-rabbit IgG (sc-2054; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as secondary antibodies (1/10 000). Immunoreactive bands were visualized with the Enhanced Chemiluminescence Detection System (Amersham Pharmacia Biotech, Orsay, France).

Luciferase assay. Jurkat cells (2.0×10^5) in 12-well plates were cotransfected with the expression vector pH β Pr-1-neo encoding Tax, Tax Δ C, TaxM22, Tax703, Tax(225–232) or Tax2B, together with pGK/ β -gal and either κ B-luc or CRE-luc using TransFectin (Bio-Rad Technologies) according to the manufacturer's instructions. At 48 h after transfection the cell lysates were prepared, and the luciferase and β -galactosidase activities in the lysates were measured using both the Luciferase Assay System (Promega, Fitchburg, WI, USA) and the Galacto-Light System (Applied Biosystems, Foster City, CA, USA), respectively.

Statistical analysis. Differences in the gene expression levels between the cell lines were analyzed using unpaired Student's *t*-tests, and were presented as the means \pm SD.

Results

Kurosawa *et al.*⁽¹⁸⁾ showed that the BCL11B expression levels in HTLV-1-infected cells were downregulated relative to those in HTLV-1-uninfected cells. To elucidate the mechanism responsible for this, we measured the expression of BCL11B in various human T-cell lines, including HTLV-1-transformed and ATL-derived cell lines by performing western blot analyses and RT-PCR assays. Consistent with the previous study, the amounts of BCL11B protein and mRNA in the HTLV-1-transformed and ATL-derived cell lines were lower than those in HTLV-1-uninfected cell lines (Fig. 1). The extent of BCL11B mRNA downregulation in HTLV-1-transformed cells, except for the MT-4 cells, was higher than that in the ATL cells. While the ATL-derived cells expressed the HBZ protein at a level equivalent to that in HTLV-1-transformed cells, they expressed no or very little Tax protein (Fig. 1a), which is typical of ATL-derived cell lines. Therefore, the present results indicate that Tax, either alone or together with HBZ, regulates BCL11B expression in HTLV-1-infected cells.

To assess this possibility, we transiently expressed Tax and the spliced form of HBZ (sHBZ) in an HTLV-1-uninfected T-cell line (Jurkat) expressing a relatively high amount of BCL11B by using lentiviral vectors (Fig. 2a,b). Tax reduced the amount of BCL11B protein in the Jurkat cells (Fig. 2b), whereas sHBZ had little effect on its expression amount (Fig. 2a). Tax also reduced the amount of BCL11B protein in another human T-cell line, MOLT-4 (Fig. 2c). The RT-PCR analysis elucidated that Tax represses BCL11B expression at the mRNA level in Jurkat cells (Fig. 2d). These results indicated that Tax represses the expression of BCL11B in HTLV-1-infected T-cells.

To explore the mechanism by which Tax regulates BCL11B repression, we transiently expressed several Tax mutants in Jurkat cells using lentiviral vectors. Similar to the results obtained for wild type Tax, four Tax mutants (Tax Δ C, TaxM22, Tax703 and Tax[225–232]) reduced the expression of the BCL11B protein in Jurkat cells, whereas Tax(TTG), a Tax mutant without an initiation codon, did not show such reduction. Moreover, HTLV-2 Tax2 also reduced the expression of the BCL11B protein. A luciferase reporter assay showed that Tax, Tax Δ C and Tax(225–232) equivalently activated the promoter activity under the control of κ B-enhancer as well as the CRE (c-AMP responsive element) enhancer in the HTLV-1 promoter (Fig. 3c,d). In contrast, TaxM22 only slightly stimulated the NF- κ B-dependent transcription. Tax703 activated CRE, but it was less effective than Tax. It should also be noted that Tax(225–232) has a selective defect for non-

Fig. 1. The downregulation of BCL11B expression in HTLV-1-infected T-cell lines. (a) Cell lysates were prepared from the indicated human T-cell lines, and the expression levels of BCL11B, Tax, HBZ and α -tubulin in the cell lysates were measured by western blot analysis. (b) Total RNA was prepared from the indicated human T-cell lines, and the amounts of BCL11B mRNA were measured by quantitative real-time RT-PCR. The quantity of BCL11B mRNA was normalized to the quantity of GAPDH mRNA. * $P < 0.05$. ATL, adult T cell leukemia; HTLV-1, human T-cell leukemia virus type 1; NS, not significant.

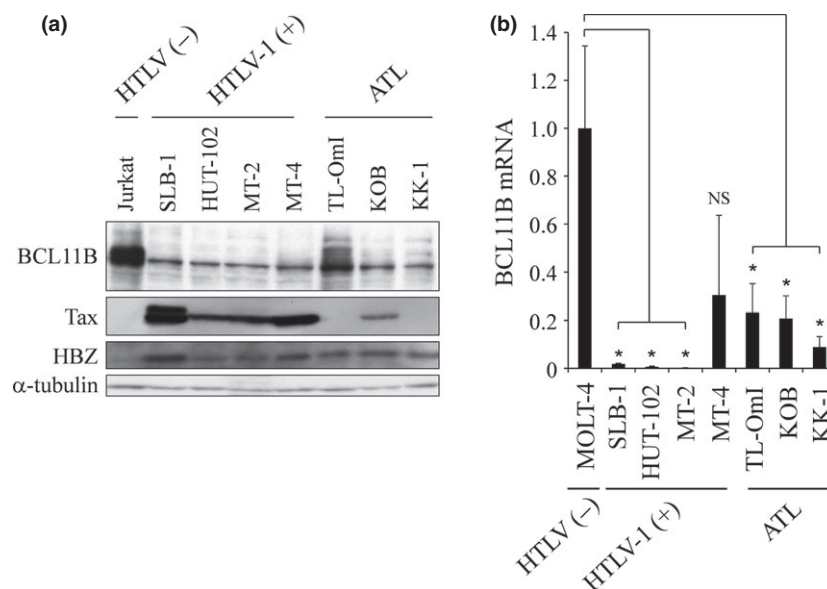
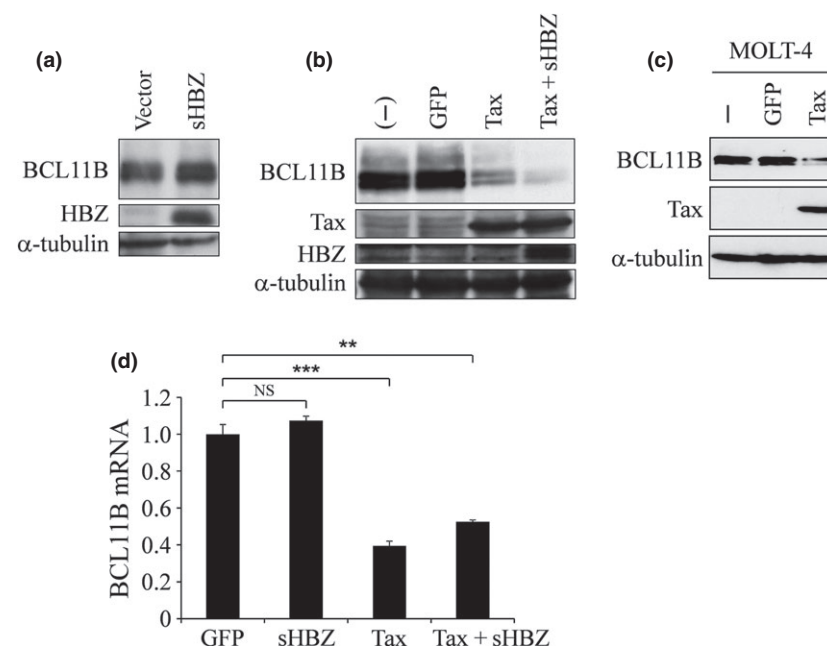


Fig. 2. Tax represses BCL11B expression in T-cells. (a–c) Cell lysates were prepared from Jurkat (a, b) or MOLT-4 cells (c) infected with the indicated lentiviruses, and the expression of BCL11B, Tax, HBZ and α -tubulin in the cell lysates was examined by western blot analysis. A lentivirus expressing the GFP protein (GFP) was used as a control. (d) Total RNA was prepared from Jurkat cells infected with the indicated lentiviruses, and the amounts of BCL11B mRNA in the cells were measured by quantitative real-time RT-PCR. The quantity of BCL11B mRNA was normalized to the quantity of GAPDH mRNA. ** $P < 0.01$; *** $P < 0.001$. NS, not significant.



canonical κ B-dependent transcriptional activation,⁽¹⁹⁾ and Tax Δ C has a deletion of the PDZ-domain binding motif (PBM).⁽²⁷⁾ These results suggested that the κ B-dependent and CRE-dependent transcriptional activation, as well as PBM-mediated signaling of Tax, are dispensable for the repression of BCL11B.

Discussion

The previous study showed that both HTLV-1-transformed T-cells and ATL-derived cell lines have reduced expression of BCL11B.⁽¹⁸⁾ In this study, we found that Tax is a major factor associated with the BCL11B downregulation in HTLV-1-transformed T-cells, and this downregulation mainly occurred at the transcriptional level. Together with the known roles of BCL11B in normal T-cell development and as a tumor suppressor in T-cell leukemia, the present study suggests that

the downregulation of BCL11B by Tax plays a role in T-cell transformation by HTLV-1 and persistent HTLV-1 infection.

How does the Tax-induced downregulation of BCL11B alter the phenotypes of HTLV-1-infected cells? Kurosawa *et al.*⁽¹⁸⁾ showed that the exogenous expression of BCL11B in two ATL-derived cell lines reduced their growth, but the expression did not affect the growth of Jurkat cells (a reduced level of the BCL11B protein is associated with adult T-cell leukemia/lymphoma). These results suggest that the downregulation of BCL11B can specifically promote the growth of HTLV-1-infected cells.

Like HTLV-1 Tax, the HTLV-2 Tax2 protein repressed the expression of the BCL11B protein (Fig. 3a). Tax2 is essential for the immortalization of primary human T-cells by HTLV-2 *in vitro*.⁽²⁸⁾ Thus, the BCL11B repression by Tax2 proteins may also play a role in HTLV-2 immortalization of T-cells. Tax and Tax2 share many activities, including NF- κ B

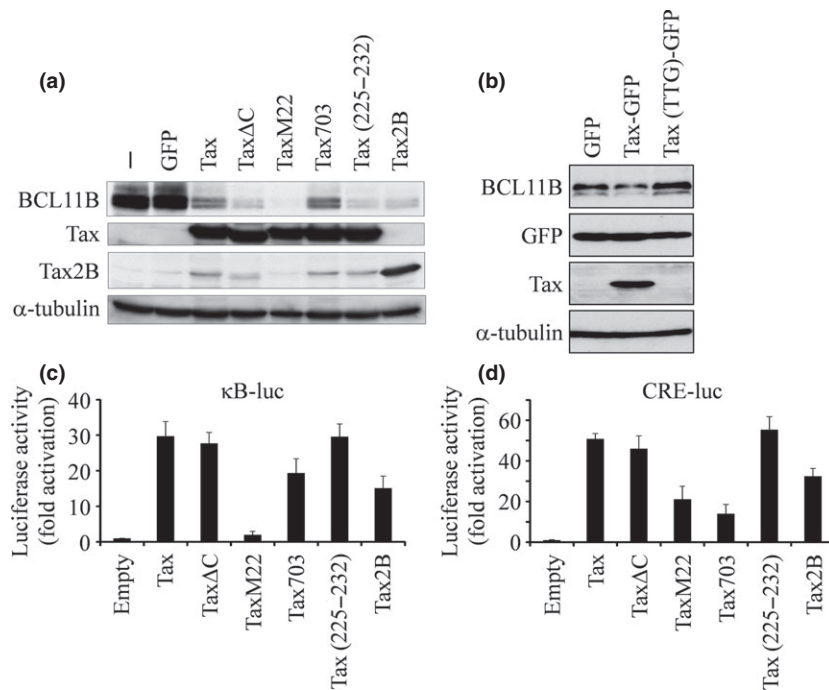


Fig. 3. Tax mutants also reduced the expression of BCL11B in T-cells. (a, b) Cell lysates were prepared from Jurkat cells infected with the indicated lentiviruses, and the expression levels of BCL11B, Tax, Tax2B, GFP and α -tubulin in the cell lysates were measured by western blot analysis. A lentivirus expressing the GFP protein (GFP) was used as a control. Tax and Tax(TTG) lentiviruses coexpressed Tax and GFP protein by a IRES-mediated bicistronic transcript (b). (c, d) Jurkat cells (2.0×10^5) were cotransfected with the indicated expression vector encoding Tax or its mutant together with pGK/ β -gal and either κ B-luc (c) or CRE-luc (d) using TransFectin. At 48 h after transfection, the cell lysates were prepared, and then the luciferase and β -galactosidase activities in the lysates were measured. The luciferase activity was normalized by the activity of β -galactosidase, and the fold activity indicates the normalized luciferase activity of Tax-transfection relative to that of the control plasmid transfection.

activation.⁽²⁹⁾ The four Tax mutants examined herein still repressed the expression of the BCL11B protein in T-cells (Fig. 3). Therefore, a further analysis is required to determine how Tax represses the expression of BCL11B in T-cells.

The BCL11B gene shows T lineage-specific expression, including CD4-positive T cells. Li *et al.*⁽³⁰⁾ identified the long-range tissue-specific transcriptional regulatory elements in the BCL11B gene, and showed that both the promoter region containing a transcription initiation site and the 1.9 kb region 850 kb downstream of the BCL11B gene are required for T lineage-specific expression. The 1.9 kb region in the BCL11B gene contained several putative transcription factor binding sites, and these TCF-1 binding sites were required for the T lineage-specific expression of BCL11B in the reporter assay.

While the three ATL cell lines possessed reduced amounts of BCL11B expression, one out of three ATL cell lines expressed a low amount of Tax protein, indicating that the downregulation of BCL11B expression in ATL cells is generally Tax-independent. Kurosawa *et al.*⁽¹⁸⁾ failed to detect DNA methylation of the BCL11B locus. Therefore, the mechanism underlying BCL11B downregulation in ATL cells remains to be elucidated.

Several leukemogenic events have been shown to be initially set in motion by Tax in HTLV-1-infected cells *in vivo*, but once such cells become transformed, then a Tax-independent mechanism may subsequently continue the leukemic process. For instance, primary ATL cells and ATL-derived cell lines

without Tax expression demonstrate constitutive NF- κ B activation, which is critical for the growth of leukemic cells and is a later stage step in the development of leukemia. Therefore, the downregulation of BCL11B by Tax in HTLV-1-infected cells sets the stage for development of ATL. Our data reveal a novel mechanism of Tax in repressing the expression of a tumor suppressor, BCL11B, in order to promote the continuous proliferation of HTLV-1-infected cells. Taken together, our findings support the idea that the downregulation of BCL11B plays a role in ATL development.

Acknowledgments

The authors thank Renaud Mahieux, William Hall, Hiroyuki Miyoshi and Toshio Kitamura for providing us with reagents. We thank Takeda Pharmaceutical Company for providing recombinant human IL-2. We also express our gratitude to Misako Tobimatsu for technical assistance. This work was supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Grant for the Promotion of Niigata University Research Project to MF and by a grant from the National Institutes of Health (CA100730) to PLG.

Disclosure Statement

The authors have no conflict of interest to declare.

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